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Purification of foetal steroid-binding protein from human serum by affinity chromatography on 5α-androstane-3β,17β-diol 3-hemisuccinate–aminohexyl-Sepharose 6B

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In order to develop an immunoassay for foetal steroid-binding protein in human serum, which is impossible to assay quantitatively in normal samples by conventional ligand-binding techniques, the protein was purified by salt precipitation, affinity chromatography and gel filtration. Elution was by competing ligand or alkaline pH. The purified protein was further characterized and a highly specific antiserum was raised in rabbits.

INTRODUCTION

This paper describes the purification and further characterization of the newly discovered foetal steroid-binding protein (FSBP), for which an e.l.i.s.a. has been developed. The major problem of purification of FSBP from human serum by specific affinity chromatography with a steroid linked to the matrix is interference from sex-hormone-binding globulin (SHBG), which has very similar steroid-binding specificity. The difference between the two proteins in pH-dependent binding was utilized in the purification of FSBP. After $(NH_4)_2SO_4$ precipitation this protein was selectively immobilized on 5α-androstane-3β,17β-diol 3-hemisuccinate–aminohexyl-Sepharose 6B at pH 5, and eluted at pH 11. The protein was purified to homogeneity by a further gel-filtration step. This purification by differential pH elution could not, however, be applied to SHBG, because of irreversible denaturation. For purposes of comparison, purification of both proteins was achieved by elution by competing ligand. The purified preparation of FSBP was used to raise a highly specific polyclonal antiserum for e.l.i.s.a.

FSBP, a sex-steroid-binding protein (Wilkinson *et al.*, 1983; Iqbal *et al.*, 1983, 1984) present in normal plasma (Iqbal *et al.*, 1985), is distinct from the other circulating oestrogen- and androgen-binding protein SHBG (Iqbal *et al.*, 1983, 1985). Its binding parameters cannot be determined in unprocessed serum (Wilkinson *et al.*, 1983; Iqbal *et al.*, 1984), and this makes calculation of a purification factor impossible. For this reason a simultaneous purification of SHBG from the same source may provide a rough guide to the losses of both proteins during the early stages of the fractionation procedure.

The closely similar steroid-binding specificity of FSBP and SHBG, differing only in the inability of FSBP to bind the 3β-epimer of 5α-androstanediol (Wilkinson *et al.*, 1983; Iqbal *et al.*, 1983), presented theoretical problems in applying an affinity-chromatography technique to their purification. However, 5α-androstane-3β,17β-diol 3-hemisuccinate–aminohexyl-Sepharose 6B (androstanediol–Sepharose) was employed because of its relatively easy manufacture, because its characteristics are already known (Iqbal *et al.*, 1978; Iqbal & Johnson, 1979) and because preliminary stabilization studies of both proteins had suggested that changes of pH during immobilization/elution of the two might allow selective purification of either SHBG or FSBP. For starting material late-pregnancy serum was taken, in which both SHBG and FSBP are known to be present in high concentrations (Iqbal *et al.*, 1978, 1985).

MATERIALS AND METHODS

Materials

17β-Hydroxy-[1,2,4,5,6,7-^{3}H]5α-androstan-3-one-([^{3}H]DHT) (specific radioactivity 104 Ci/mmol) was purchased from Amersham International. Sepharose 4B, concanavalin A–Sepharose 4B, Sepharose 6B, Sephadex LH-20 and Sephadex G-150 were from Pharmacia (G.B.). Cibacron Blue 3GA–Sepharose 6B was prepared by the method of Heyns & De Moor (1974). PM-10 membranes and Minicon B-15 micro concentrator cells were purchased from Amicon (U.K.). All other chemicals were of analytical grade.

Assay of binding capacity of FSBP and SHBG

The two-tier column method was employed for measuring binding capacity for DHT of SHBG (Iqbal & Johnson, 1977) and FSBP (Wilkinson *et al.*, 1983; Iqbal *et al.*, 1983) throughout the purification, the former being measured in the column eluates and the latter by determination of radioactivity in the Cibacron Blue 3GA–Sepharose 6B (Blue gel) portion of the column. The following modifications were used. pH stability studies after a pilot purification (see below) had demonstrated that FSBP binding could be measured conveniently at pH 5 (SHBG binding is minimal at this pH), whereas at pH 7.5 FSBP binding cannot be quantified in untreated sera (Iqbal *et al.*, 1983, 1984) although SHBG binding is maximal. The FSBP assays in

Abbreviations used: FSBP, foetal steroid-binding protein; SHBG, sex-hormone-binding globulin; DHT, 17β-hydroxy-5α-androstan-3-one; e.l.i.s.a., enzyme-linked immunosorbent assay.

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the purification procedure was therefore carried out at pH5 and those for SHBG at the standard pH 7.5. After the affinity step, when protein concentrations were below those required for the assay, samples to be assayed were made up in 1:1 dilutions of serum previously treated at pH 7.5 with androstanediol–Sepharose to remove SHBG and FSBP and with Blue gel to remove albumin and any remaining FSBP. Binding to DHT after the gel-filtration step was determined by the charcoal separation technique (Iqbal & Johnson, 1976).

Differential stability of FSBP and SHBG

Late-pregnancy serum was obtained from over 1000 women in the last trimester of pregnancy, stored at −20 °C until thawed, pooled and assayed as below without refreezing. Effects of charcoal, EDTA, heat, pH, Ca^{2+}, glycerol, GSSG and GSH on denaturation of both proteins have been studied (Wilkinson, 1985). Only Ca^{2+} and glycerol have been found to protect both proteins from heat-denaturation, and pH-dependent binding differed markedly between the two proteins in that FSBP concentrations were highest at pH 5 and those of SHBG at pH 11.

Purification of FSBP and SHBG

(a) $(NH_4)_2SO_4$ precipitation. To 1.5 litres of pooled late-pregnancy serum was added 469.5 g of solid $(NH_4)_2SO_4$, and the mixture was stirred vigorously for 1 h on a magnetic stirrer before centrifugation at 20000 *g* for 30 min at 4 °C. The precipitate was washed in 2.025 M-$(NH_4)_2SO_4$, re-centrifuged and the resultant precipitate dissolved in 1.5 litres of Tris buffer (50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-$CaCl_2$).

(b) Affinity immobilization and elution with competing ligand. The solution from the above step was gently stirred for 2 h with 100 ml of androstanediol–Sepharose, prepared as previously described (Iqbal & Johnson, 1979), at pH 7.5 before filtration in a coarse sintered-glass funnel. The gel was then washed with 2.5 litres of Tris buffer in 100 ml portions until the protein content of the eluate was negligible. A further washing with four 100 ml portions of 1 M-NaCl in the same buffer until protein losses were negligible was followed by an additional four 100 ml portions of Tris buffer.

Elution was performed with 400 ml of 0.07 mM-DHT in Tris buffer, in 100 ml portions added at room temperature over a period of 1 h. The pooled eluate was concentrated to 3 ml, first by using an Amicon ultrafiltration concentrator and subsequently by using the Amicon (Minicon B-15) micro concentrator cell. It was applied to a pumped pre-calibrated 2.5 cm × 104 cm column of Sephadex G-150. The absorbance at 280 nm was monitored and the two protein peaks obtained were assayed for FSBP and SHBG. The presence of the two proteins was also determined by polyacrylamide-gel electrophoresis with the use of 10% total gel with 2.5% cross-linkage, and with late-pregnancy serum and SHBG previously purified to homogeneity as controls, by the method of Smith (1968). The proteins were tested for homogeneity by SDS/polyacrylamide-gel electrophoresis by the method of Weber & Osborn (1969) with and without prior treatment with 2-mercaptoethanol at 80 °C for 5 min and at 45 °C for 2 h.

(c) Affinity immobilization and differential pH elution. $(NH_4)_2SO_4$ precipitation was performed on 2.5 litres of pooled late-pregnancy serum as described above. The affinity immobilization step was carried out as before but at pH 5 to immobilize FSBP selectively. Washes with Tris buffer and NaCl/Tris buffer were performed as above but at pH 5. Elution took place with 400 ml of 50 mM-Tris/384 mM-glycine/HCl buffer, pH 11, at which FSBP binding is minimal, the pH of the eluate being immediately adjusted to pH 7.5. After concentration of the eluate in Amicon cells, polyacrylamide-gel electrophoresis was carried out as above on both the affinity supernatant and the eluate. No binding of SHBG could be detected by the two-tier column method in either the affinity supernatant or the eluate, even after adjustment of the pH to 7.5. Analytical polyacrylamide-gel electrophoresis carried out on supernatant and eluate failed to show SHBG in the eluate, but a band corresponding to the SHBG in the control samples was detected in the supernatant.

The affinity gel from the immobilization step in subsection (*b*) above was subsequently treated with Tris/glycine buffer at pH 11 to remove any remaining FSBP, after washes with Tris buffer and NaCl/Tris buffer at pH 7.5. This sample was concentrated in an Amicon cell with a PM-10 membrane before assay for FSBP and SHBG and polyacrylamide-gel electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed on both pH-dependent eluates as described above.

Purification of SHBG

SHBG was also purified by the technique of Iqbal & Johnson (1979), but with the single modification that the eluting buffer contained 150 mM-DHT in 20% (v/v) dimethylformamide made up in the buffer.

Protein concentrations were monitored at each stage of the purification procedure by using the method of Lowry *et al.* (1951), with human serum albumin as standard.

Characterization of SHBG and FSBP

(a) Sucrose-density-gradient ultracentrifugation. This was performed, by the method of Hansson *et al.* (1972), on purified FSBP and purified SHBG samples pre-incubated with [^{3}H]DHT (20000 d.p.m.), in 10.5 ml ultracentrifugation tubes containing linear 5–20% (w/v) sucrose gradients in Tris buffer; 200 μl samples were applied and the tubes were centrifuged at 40000 *g* for 18 h at 4 °C before collection of 210 μl fractions. The absorbance at 280 nm and radioactivity were monitored. Human serum albumin was run as a standard.

(b) Competition studies. Purified FSBP, 2 μg diluted in 20 ml of androstanediol–Sepharose-treated Blue-gel-treated serum (i.e. serum free from albumin, SHBG and FSBP) was employed for these studies. Portions (0.4 ml) were incubated with a constant amount of [^{3}H]DHT (30000 d.p.m.) and various amounts (0–350 pmol) of unlabelled DHT, androst-5-ene-3β,17β-diol, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol, testosterone, oestradiol, cyproterone, cyproterone acetate, progesterone or cortisol. The two-tier column assay for FSBP was carried out at pH 7.5 as above.

Table 1. Purification of FSBP by affinity chromatography on androstanediol–Sepharose

For experimental details see the text.

(*a*) Elution by competing ligand and subsequently by high-pH buffer

	Original serum	$(NH_4)_2SO_4$ fractionation	Elution from affinity gel: By DHT	Elution from affinity gel: By pH 11 buffer	Sephadex G-150 filtration
Total binding capacity (nmol)	334.5	315	20.0	3.15	—
Total protein (mg)	112800	47250	2.205	0.9405	1.376
Specific activity (nmol/mg)	0.002965	0.006667	2.068	3.349	—
Purification factor (fold)		2.25	3058	1130	—
Recovery (%)		94.2	5.98	0.942	—

(*b*) Elution by high-pH buffer alone

	Original serum	$(NH_4)_2SO_4$ fractionation	Elution from affinity gel by pH 11 buffer
Total binding capacity (nmol)	967.5	902.5	10.76
Total protein (mg)	202500	82750	2.0
Specific activity (nmol/mg)	0.0478	0.0109	107.6
Purification factor (fold)		2.28	2251
Recovery (%)		93.3	22.3

Raising of polyclonal antibodies to FSBP and SHBG

Purified FSBP and SHBG were injected into female New Zealand White rabbits together with Freund's adjuvant at monthly intervals for the raising of specific antibodies to the two proteins. Sera were screened by double radial immunodiffusion (Ouchterlony, 1958) against normal male and pregnancy sera, against purified FSBP and against purified SHBG.

RESULTS

The capacity of androstanediol–Sepharose for SHBG was 25 ml of pregnancy serum pool or 5.58 nmol (DHT-bound) of SHBG per ml of gel.

Purification of FSBP and SHBG elution by competing ligand

Recovery of SHBG in the $(NH_4)_2SO_4$ step was 94.2% (Table 1) with a 58% decrease in protein concentration. The affinity immobilization and elution with competing ligand produced a yield of FSBP of almost 6% [assuming losses in the $(NH_4)_2SO_4$ step to be the same as those of SHBG] and a purification for this protein exceeding 3000-fold. When elution of the same gel with a pH 11 buffer was performed, a further 0.94% yield was obtained with a lower purification factor but a high association constant (approx. 10^9 M^{-1}) (Table 1). On application of this sample to the Sephadex G-150 column, two protein peaks corresponding to M_r 54000 (FSBP) and 83000 (SHBG) were detected. However, binding studies detected only non-specific binding for the former and an affinity of 4.5×10^7 M^{-1} for the latter.

Polyacrylamide-gel electrophoresis of the DHT eluate showed two bands corresponding to R_F values of 0.29 and 0.44, the major steroid binding being produced by the former, corresponding to the known R_F of SHBG under these conditions, although binding at the second band was also detectable. After elution by pH 11 buffer, only one band was seen on SDS/polyacrylamide-gel electrophoresis, corresponding to an M_r of 70000 ± 500, though after 2-mercaptoethanol treatment a band was seen at M_r 71000 and another at M_r 47000 (Fig. 1).

Purification of FSBP by employing differential pH elution for the major elution step

Similar results were obtained with the $(NH_4)_2SO_4$ precipitation step. After affinity immobilization at pH 5

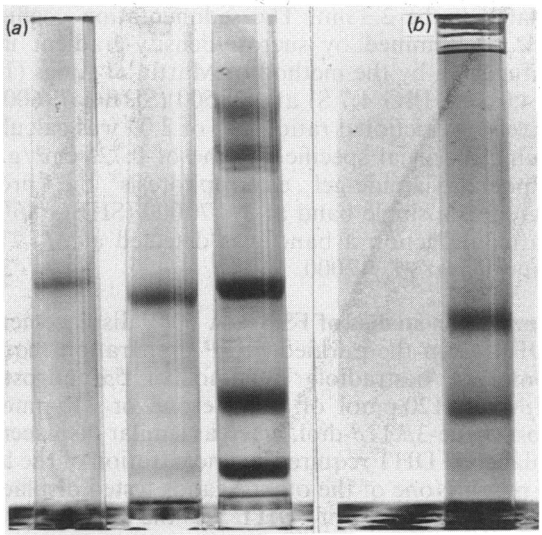

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified FSBP

(*a*) Purified protein from pH-dependent (first gel) and competing-ligand (second gel) elutions and protein standards (third gel). (*b*) Purified protein after 2-mercaptoethanol reduction. The five major bands in the control gel are, from top to bottom, as follows: cross-linked albumin, M_r 272000; cross-linked albumin, M_r 136000; human serum albumin, M_r 68000; ovalbumin, M_r 45000; carbonic anhydrase, M_r 29000.

Table 2. Purification of SHBG

For experimental details see the text.

	Total protein (mg)	Total binding capacity (nmol of DHT bound)	Specific activity (nmol/mg)	Recovery (%)	Purification factor (fold)
Original serum	38750	50	0.00129	—	—
Final preparation	0.684	8.55	12.5	24.4	9690

no SHBG binding activity could be detected in the affinity supernatant or eluate, although a band corresponding to SHBG in the control samples was seen on polyacrylamide-gel electrophoresis of the supernatant. FSBP was detectable in the supernatant by binding and by polyacrylamide-gel electrophoresis. The specific activity and recovery of the pH-dependent elution were poorer than by the elution by competing ligand, the association constant being 1.5×10^7 M^{-1} (Table 1).

Purification of SHBG

SHBG purified by the technique of Iqbal & Johnson (1979), but with a high concentration of DHT in 20% dimethylformamide produced a high yield and high purification factor (Table 2).

Characterization of FSBP and SHBG

Determination of M_r. By reference to standard proteins used to calibrate the column (bovine serum albumin, catalase, ovalbumin and cytochrome *c*) the M_r of FSBP on Sephadex G-150 gel filtration was 54000 (that of SHBG 83000). In accordance with the method of Laurent & Killander (1964), the Einstein–Stokes radius was calculated to be 2.3 nm. The sedimentation coefficient and M_r determined by sucrose-density-gradient ultracentrifugation by the method of Martin & Ames (1961) were 4.0 S (SHBG 4.7 S) and 38500 (SHBG 79600) respectively. A frictional ratio, f/f_0, of 2.09 was calculated assuming a partial specific volume of 0.725 cm^3/g. On SDS/polyacrylamide-gel electrophoresis the protein appeared as a single band at M_r 70000 (SHBG 86000), and after reduction a band was detected at M_r 71000 and another at M_r 47000.

Competition studies of FSBP. A 50% displacement of [^{3}H]DHT from the purified FSBP preparation required 180 pmol of oestradiol, 130 pmol of 5α-androstane-3α,17β-diol, 120 pmol of testosterone or 115 pmol of androst-5-ene-3β,17β-diol, whereas similar displacement by unlabelled DHT required a concentration of the latter of 35 pmol. None of the other ligands tested displaced a significant quantity of DHT at the concentrations employed.

Antibodies to FSBP and SHBG

After five immunizations with purified FSBP one rabbit gave a high-titre polyclonal antibody to FSBP that showed no cross-reactivity with SHBG or albumin on double immuno-diffusion. An e.l.i.s.a. for FSBP was subsequently developed (Iqbal *et al.*, 1985), SHBG antibody was detected from two rabbits after five immunizations, but an e.l.i.s.a. for this protein has not yet been developed.

DISCUSSION

The differences demonstrated in pH-dependent binding between the two proteins enabled separation of FSBP and SHBG without a gel-filtration or other preparative step. However, the findings in the stabilization studies were not entirely applicable to the purification procedure, as in these circumstances the denaturation of SHBG was such as to preclude its purification by the pH-dependent technique.

In previous purifications of androgen-binding proteins by affinity chromatography, either SHBG or the androgen receptor, the major problems have been in designing a gel that had a sufficiently high binding affinity and capacity for the protein but that did not prevent elution of the major portion of the protein in active form. Although the use by the group of Mickelson and Petra (Mickelson & Petra, 1975; Mickelson *et al.*, 1978) of DHT linked to a gel via the C-17 position diminished binding affinity to SHBG 600-fold, elution was correspondingly simpler. In the present purification, the use of androstanediol–Sepharose meant that the major difficulty in the purification process was likely to be in elution rather than immobilization. The additional problem presented by the need to separate two proteins of closely similar binding characteristics was approached in a manner that has not previously been applied successfully to the binding proteins. Indeed, SHBG is irreversibly denatured at pH 3 (Cuatrecasas, 1970). Although FSBP survived immobilization at acid pH, namely pH 5, and SHBG was not irreversibly denatured at this pH in solution, the apparent difference in behaviour of SHBG when in the presence of the affinity gel is not fully explained. In subsequent small-scale purifications FSBP is rapidly and conveniently prepared by a combination of $(NH_4)_2SO_4$ fractionation and immobilization on androstanediol–Sepharose with elution at pH 11, the whole procedure being completed within a working day. SHBG can also be rapidly and conveniently prepared by a combination of $(NH_4)_2SO_4$ precipitation, Blue-gel treatment, affinity immobilization on androstanediol Sepharose at pH 7.5 and elution by 150 mM-DHT in buffer containing 20% (dimethylformamide (Table 2). This approach cannot be adopted for FSBP, as no comparable method of removing SHBG is available.

The discrepancies between M_r values and amino acid and carbohydrate compositions of SHBG purified on the one hand by Rosner & Smith (1975), Iqbal & Johnson (1978, 1979) and Mercier-Bodard *et al.* (1979) and on the other by the group of Mickelson and Petra (Mickelson & Petra, 1975; Mickelson *et al.*, 1978; Petra, 1979), together with the marked differences in M_r found for SHBG by the latter group of workers using different

analytical techniques, might suggest that Mickelson and colleagues have on at least some occasions purified FSBP in addition to or instead of SHBG. FSBP seems to behave in a similar manner with different analytical techniques and has a similar M_r on sucrose-density-gradient ultracentrifugation to the protein described by Mickelson & Petra (1975).

The binding specificity of FSBP indicated by the competition studies with the purified protein confirms the findings in unpurified foetal liver cytosol (Wilkinson *et al.*, 1983) and hepatocellular carcinoma serum (Iqbal *et al.*, 1983). In particular the lack of binding to the 3β-epimer of 5α-androstanediol is repeated. From these data and their lack of immunological cross-reactivity it is clear that FSBP and SHBG are distinct sex-steroid-binding proteins.

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